Reduced NGF level and TrkA protein and TrkA gene expression in the optic nerve of rats with experimentally induced glaucoma

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ABSTRACT

Glaucoma (GL) is an optic neuropathy characterized by progressive loss of visual field due to retinal cell death and optic nerve (ON) degeneration, usually in response to abnormal elevated intraocular pressure (EIOP). It has previously demonstrated that cells of the ON express nerve growth factor (NGF) and NGF-receptors. Relatively little is known, however, about their role on the ON of the glaucomatous eye. The aim of the study was to elucidate this aspect. Using a rat model of GL we investigated the response of NGF and NGF-receptors in the ON of subjects with experimentally induced EIOP. Our results show that EIOP significantly impairs the presence of NGF and NGF-receptor proteins and TrkA gene expression in the ON of glaucomatous eye. These findings suggest that NGF and NGF-receptor might be important signals for the ON response in the EIOP.

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Glaucoma (GL) is an ocular disorder characterized by progressive loss of visual field due to retinal ganglion cell (RGC) death and degeneration of the optic nerve (ON) fibers, usually in response to elevated intraocular pressure (EIOP) [23]. There is, however, evidences that altered environmental factors can contribute and/or exacerbate these degenerative events [11,25,27]. The neurotrophins, that include nerve growth factor (NGF), brain-derived neurotrophic factor (BDNF) and neurotrophin-3 (NT-3), are endogenously produced signaling molecules that play marked roles on specific cell population of peripheral sympathetic and sensory system and of brain neurons [3,9,19] and, as it has been demonstrated in a number of recent studies, also on cells of the visual system [7,18,26]. For example, NGF has been shown to exert a protective action on damaged ON of fish [6], amphibians [29], birds [10], and mammals [7,11,12], while abnormal expression of NGF, NGF-receptor proteins and of its mRNA has been detected in the ON of post-mortem subjects with multiple sclerosis [20] and in a rat model [2]. The functional role of NGF in the ON of glaucomatous eye is still largely unknown. Because GL induces marked damages in fibers and in cells of the ON cells, we investigated the

response of NGF and NGF-receptors in the ON of experimentally induced GL in adult rats. Our results show that EIOP alters significantly the expression of NGF and the high-affinity NGF-receptor TrkA. These findings suggest that NGF and NGF-receptor are important signaling molecules for the ON and most likely for retinal cells.

Animal care and handling were in compliance in conformity with National and International laws (EEC Council Directive 86/609, OJ L 358, 1, December 12, 1987) and according to the ARVO Statement for the Use of Animals in Ophthalmic and Vision Research.

Unilateral elevation of IOP was induced in right eye of anesthetized adult male Sprague Dawley rats ($n = 20$) by injecting $50 \mu l$ of hypertonic saline into the superior episcleral vein, as described [21]. The left eye was used as non glaucomatous control rats. All experimental rats were housed in a constant low-light environment (40–90 lx) to minimize IOP circadian oscillations. Intraocular pressure was determined in awake animals using one drop of 0.5% proparacaine hydrochloride instilled in each eye. The calibrated TonoPen XL tonometer (Mentor, Norwell, MA) was used for daily monitoring of IOP. Animals with an IOP less than 30 mmHg were not classified as glaucomatous rats and were not used for this study. All rats were killed 5 weeks after induction of GL and retina and eye globe with ON removed and used for morphological, immunohistochemical and molecular analysis.

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The concentration of NGF was measured by a highly sensitive two-site enzyme immunoassay ELISA kit "NGF Emax^{TM} Immunoassay System number G7631" by Promega (Madison, WI, USA), following the instructions provided by the manufacturer. The amount of NGF was determined from the regression line for the NGF standard (ranging from 7.8 to 500 pg/ml of purified mouse NGF), incubated under similar conditions in each assay and all assays were performed in duplicate.

The eye globe, including the ON were fixed in Bouin's fluid for 4 days, washed several times in 10% ammonium sulfate in 80% alcohol for 3 days to remove the fixative. The ON and retina were then individually sectioned with a cryostat at −20 °C and coded sections (10 μm thick) of the ON segments 1 mm from the back of the globe and retina were used for histological and/or immunohistochemical studies.

For histological analysis retina and ON sections were stained with hematoxilin–eosin and examined with a Zeiss Axiphot microscope with an objective 40× and a final magnification of 400×.

Other sections ($n = 10$) from each glaucomatous ON ($n = 5$) and control ON ($n = 5$) were immunostained with anti-TrkA, and anti-p75 as previously described [2,15]. Briefly, sections were first exposed to 3% of hydrogen peroxide (H_2O_2) and 10% of methanol W/V for 20 min followed by exposure of 0.1 M PBS containing 10% of horse serum for 1 h and then incubated overnight at 4 °C with anti-bodies against anti-TrkA, purchased from UPSTATE (Temecula, CA, USA). Sections were then exposed to biotinylated anti-rabbit IgG 1:300 (Vector Laboratories) with 2% of horse serum for 2 h at room temperature and then with avidin-biotinylated horseradish peroxidase complex (Vector Laboratories) in 0.1 M PBS with 0.1% Triton X-100 for another 2 h at room temperature followed by the treatment for 15′ with a 0.1 M solution of 3,3′-diaminobenzidine (DAB) (Sigma Chemical Company) in 0.1 M PBS 0.1% Triton X-100 containing, 0.025% cobalt chloride, and 0.02% nickel ammonium sulfate. All the section passed through all procedures simultaneously to minimize any difference from immunohistochemical staining itself. Staining specificity was assessed by omission of the primary antibodies.

Immunostained sections were observed under a Zeiss Axiophot microscope with an objective 100× under oil immersion and TrkA-positive cells counted in randomly selected, non-overlapping fields ($n = 12$) of each ON ($n = 5$) of control and glaucomatous eye ($n = 5$). For quantitative determination, we used an image processing analysis program Nikon-Lucia that automatically selects only cell bodies, but not small fragments or cells that do not have a complete soma and expressed as the number of immune-positive cells/300 mm^2 for ON area.

For ultra structural analysis, the ON head was fixed in 4% glutaraldehyde in 0.1 phosphate buffer, pH 7.2, for 24 h, washed with fresh buffer and then post-fixed in 1% OsO4 in the same buffer. The ON was then dehydrated with ascending ethanol and acetone and embedded in 812/Spurr's low-viscosity resin. Thin sections were then cut with an ultramicrotome and collected on 200 mesh grids. Sections were stained with uranyl acetate and lead citrate and viewed under an electron microscope.

For western Blotting Analysis of TrkA and p75 receptors, tissue samples were homogenized in lysis buffer (0.01 M Tris–HCl buffer, pH 7.4, containing 0.1 M NaCl, 1 mM EDTA, 1 mM EGTA, 2 mM PMSF, 50 μM leupeptin, 100 μg/ml pepstatin, and 100 μg/ml aprotinin) at 4 °C. After 8000 × g centrifugation for 20 min, the supernatants were used for Western blotting. Samples (30 μg of total protein) were dissolved with loading buffer (0.1 M Tris–HCl buffer, pH 6.8, containing 0.2 M DTT, 4% SDS, 20% glycerol, and 0.1% bromophenol blue), separated by 8% SDS-PAGE, and electrophoretically transferred to PVDF membrane for overnight. The membranes were incubated for 1 h at room temperature with blocking buffer (5% non-fat dry milk, 10 mM Tris, pH 7.5, 100 mM NaCl, and 0.1% Tween-20). Membranes were washed three times for 10 min each at room temperature in TTBS (10 mM Tris, pH 7.5, 100 mM NaCl, and 0.1% Tween-20) followed by incubation at 4 °C with primary antibodies over night. Membranes were washed three times for 10 min each at room temperature in TTBS and incubated for 1 h with either horseradish peroxidase-conjugated anti-rabbit IgG or horseradish peroxidase-conjugated anti-mouse IgG as the secondary antibody. The blots were developed with ECL chemiluminescent HRP substrate (Millipore Corporation, Billerica, USA) as the chromophore. A personal computer and the public domain (http://rsb.info.nih.gov/ij/) Image J software were used to evaluate band density, which was expressed as arbitrary units of grey level. The ImageJ software determines the optical density of the bands using a grey scale thresholding operation. The optical density of β-actin bands was used as a normalizing factor. For each gel/blot, the normalized values were then expressed as percentage of relative normalized controls and used for statistical evaluation.

For RT-PCR analysis of TrkA and p75, total RNA from retina and ON was extracted using Versagene^{TM} RNA tissue kits (Gentra Systems Inc.). RNA was quantified by spectrophotometer at 260 nm. RNA was converted into cDNA in a 25 μl reverse transcription reaction containing 2γ of total RNA. Reactions were incubated at 42 °C for 60 min, heated at 95 °C for 5 min, then cooled at 4 °C for a minimum of 5 min and a maximum of 30 min.

The polymerase chain reaction (PCR) was analyzed using the 7900HT Fast Real-Time PCR System (PE Applied Biosystems) and FAM-labeled probe specific for the NTrk1 (NM_021589.1), and Ngfr (NM_012610.1) (PE Applied Biosystems). Designed primers and a FAM-labeled probe for rat ACTB (actin, beta) (4352931E) were included in the reactions as endogenous control. The cDNA was amplified under the following conditions: 1 cycle at 50 °C for 2 min and at 95 °C for 10 min, followed by 40 cycles at 95 °C for 15 s and at 60 °C for 1 min. The amount of mRNA of each gene was calculated using the standard curve method and adjusted for the expression of ACTB. The data have been presented as percentage of control groups.

Statistical evaluations were performed using the StatView package for Windows and data were expressed as mean and S.E.M. TrkA and p75 optical density data from Western blot analyses were evaluated using the analysis of variance (one-way ANOVA) followed by Tukey–Kramer post hoc test. A p-value less than 0.05 was considered significant.

As previously reported [2,15], intraocular administration of hypertonic saline into the episcleral vein of adult rats increases the IOP. A week after this treatment the IOP was 42.7 ± 3.7 mmHg compared to 26.9 ± 2.6 mmHg of control eye. The IOP remains elevated for 5 weeks, the last point-time checked.

Animals were killed 5 weeks following the surgical procedure. Histological analysis, shown in Fig. 1, indicated that EIOP causes reduction of cells in the retinal ganglion layer (B) and in the ON (D), compared to their respective controls, Fig. 1A and C. Moreover, detailed ultrastructural studies revealed that EIOP causes axon swelling and myelin debris, as indicated with arrows in Fig. 1E and F. Immunohistochemical studies showed the presence of TrkA stained cells in the ON, Fig. 1G and H. A marked reduction in the presence of TrkA stained cells was found in the ON in EIOP, Fig. 1H as compared to the control rats, Fig. 1G.

Quantitative analysis of immunostained TrkA-positive cells indicates that this decrease is statistically significant ($p < 0.01$), Fig. 1I. No differences in p75 immunostained cells between control and glaucomatous ON nerve were found (data not shown). The result of the immunoenzymatic assay, reported in Fig. 1L, revealed that the EIOP also causes a significant ($p < 0.01$) reduction of NGF protein in the ON.
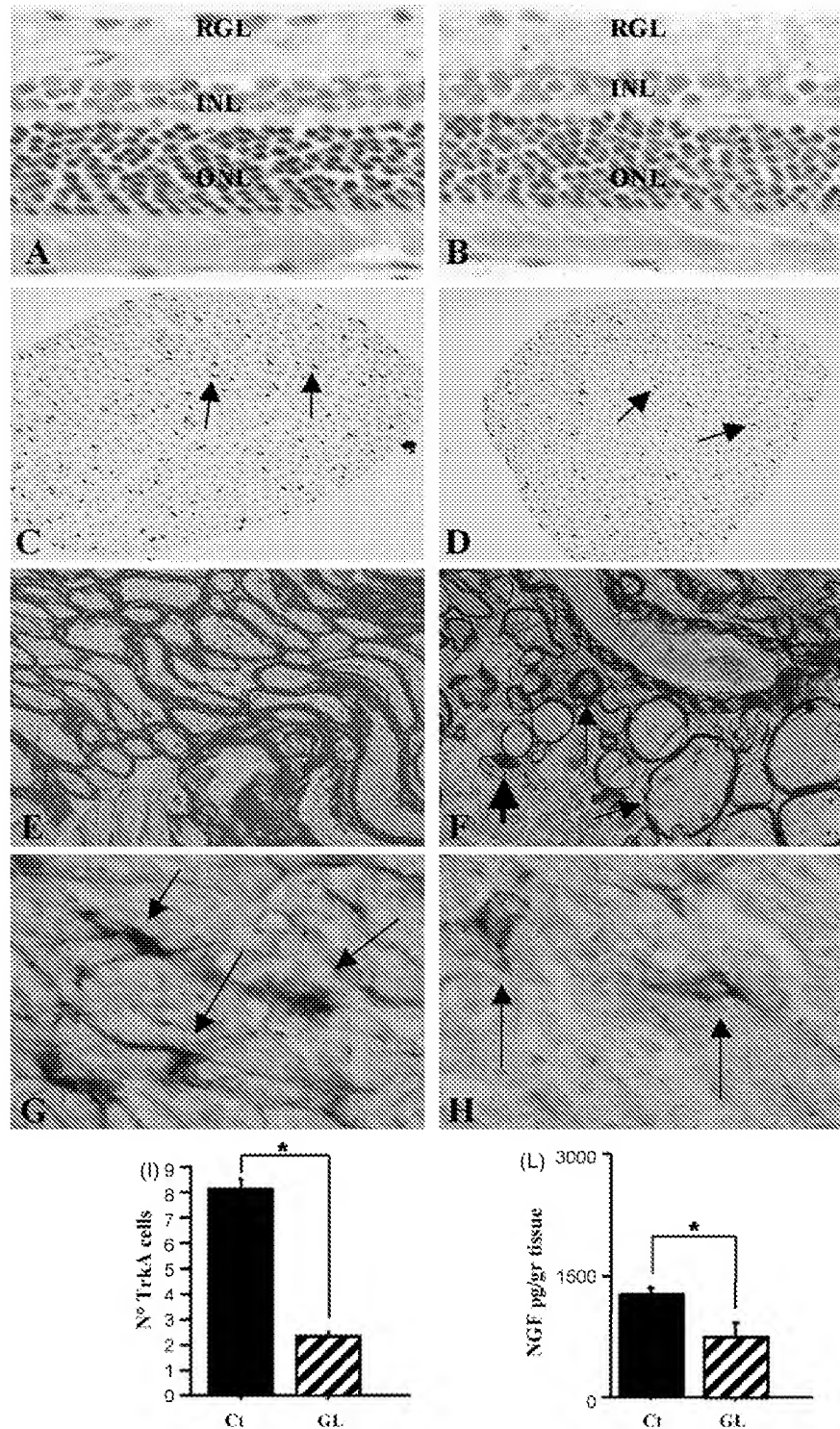
Fig. 1. Sections of control (A) and glaucomatous (B) retina showing cell reduction in the retinal ganglion layer (RGL) in the eye of rat with EIOP. INL, inner nuclear layer; ONL, outer nuclear layer. Magnification 210×. Representative histological preparations of control (C) and of glaucomatous ON (D). Note that the presence of cells (arrows) in the ON of glaucomatous rats less numerous compared to control ON. Magnification 60×. Representative electron microscopic fields of control (E) and glaucomatous ON (F) showing axon swelling (arrows), and myelin debris (thick arrow) in the glaucomatous ON. Magnification 35,000×. Representative TrkA-immunostained sections of control (G) and glaucomatous ON (H). Note the marked reduction of immunostained TrkA cells (arrows) in glaucomatous ON. Magnification 300×. (I) Quantitative evaluation of TrkA-immunostained cells in control (Ct) and glaucomatous (GL) ON. Note the statistical significant ($p < 0.01$) reduction of TrkA-positive cells in glaucomatous ON. (L) Level of NGF protein in the ON of control (Ct) and glaucomatous (GL) indicating reduced presence ($p < 0.01$) of NGF in the ON glaucomatous eye.
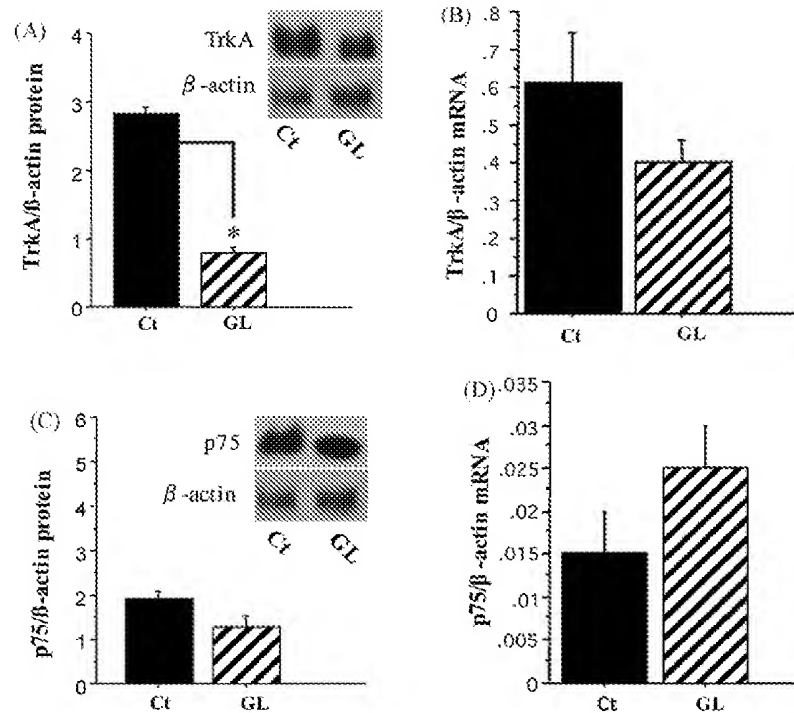
Fig. 2. TrkA protein (A) and TrkA-mRNA (B) expression in the ON of control (Ct) and glaucomatous (GL) ON. Note the marked reduction, particularly of TrkA protein in glaucomatous ON. p75 (C) protein and p75-mRNA (D) in the ON of control (Ct) and glaucomatous (GL) ON. No significant differences were found in the expression of p75 protein and gene expression, between control and glaucomatous ON.

As reported in Fig. 2, western blot and RT-PCR studies indicated that EIOP reduces the presence of TrkA protein (Fig. 2A,) and TrkA gene expression (Fig. 2B) in the ON. EIOP has no effect on p75 protein (Fig. 2C), and gene expression (Fig. 2D).

Results from the present study indicate that EIOP induced by injection of hypertonic saline into the episcleral vein reduced the presence of NGF in the ON. EIOP reduces the number of TrkA-positive cells in the ON, compared to control ON, and causes no changes in the p75 expression. Moreover biochemical and molecular analyses indicated that EIOP reduces the presence of TrkA protein and TrkA gene expression, but not p75 protein and gene expression. The effect of EIOP is associated with widespread axonal swelling in the ON, as reported by others [21]. The functional significance of the different expression in NGF-receptors in the glaucomatous eye is not clear. NGF is known to promote cell survival in the presence of TrkA, while p75 has been shown to promote apoptosis under conditions where TrkA is reduced or absent [8]. Thus, the low-affinity NGF-receptor, p75, can be involved in cell death and cell survival, depending on a number of biochemical and molecular variables. It is therefore possible that the different response of these receptors in glaucomatous ON is most likely related to these variable mechanisms. Another possibility is that within the ON, TrkA and p75 are independently regulated. For example, in the brain the expression of p75 is not associated with apoptotic mechanism [22].

The major cell components of the ON are the oligodendrocytes that play an important role in supporting the functional activity of ON axons [24]. These cells produce and release NGF [4,5,20,29] and seem to be able to regulate the synthesis and activity of NGF through autocrine mechanisms [28]. However, whether the altered NGF and TrkA expression is the result of structural changes of the ON induced by EIOP or vice versa needs to be further investigated.

The hypothesis of a functional role of NGF in the ON is supported by evidences showing that NGF and its receptors are expressed in fish, amphibian, and mammalian ON [6,10,29], including the ON

of subjects affected by multiple sclerosis [20] and that their presence is important during axonal regeneration, and damaged ON. Other findings showing that NGF promotes retinal cellular growth and differentiation [6,29,30], reduces retinal cell damage due to ocular ischemia [26], protects damaged retinal cells death induced by ocular hypertension [14] and that intraocular administration of NGF reduces RGC degeneration following rat ON lesions [7] are consistent with this hypothesis.

Based on the above observations we speculate that downregulation of the high-affinity NGF-receptor in combination with reduced presence of NGF may contribute to the progression of ON axon degeneration in glaucomatous eye. These findings may have important clinical bearing as NGF treatment previously has been applied with success in a variety of eye disorders [1,13,16,17]. Further studies are planned to investigate the effect of NGF treatment in EIOP induced ON neuropathy.

Acknowledgements

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